

Reversible threonine phosphorylation of a response regulator, SptR, modulates Group A  
*Streptococcus* (GAS, *S. pyogenes*) virulence

Honors Research Thesis

Presented in Partial Fulfillment of the Requirements for graduation  
“with honors research distinction in Microbiology” in the Undergraduate College of Natural and  
Mathematical Sciences at The Ohio State University

by  
Alisha Kamboj

The Ohio State University  
April 2014

Project Advisor: Dr. Vijay Pancholi, Department of Pathology

Reversible threonine phosphorylation of a response regulator, SptR, modulates Group A *Streptococcus* (GAS, *S. pyogenes*) virulence

Alisha Kamboj<sup>1</sup>; Rebekah Lantz<sup>1</sup>; Bhavani Gopalakrishnan<sup>2</sup>; Sashi Kant <sup>2</sup> Vijay Pancholi<sup>2</sup>

**Affiliations:** <sup>1</sup>The Ohio State University, Columbus, OH; <sup>2</sup>The Ohio State University College of Medicine, Columbus, OH

**Address correspondence to:**

Vijay Pancholi, PhD  
Associate Professor, Department of Pathology,  
The Ohio State University College of Medicine  
288 Tzagournis Medical Research Facility  
420 W 12th Ave., Columbus, OH 43210  
E-mail: vijay.pancholi@osumc.edu  
Phone: 614-688-8053

**Short Title:** Reversible threonine phosphorylation of SptR and Group A *Streptococcus* virulence

**Abbreviations**

GAS – Group A Streptococcus, *S. pyogenes*  
TCS – Two-Component Regulatory Systems  
RR – Response Regulator  
HK – Histidine Kinase  
CMT – Carbohydrate-Metabolism and Transport  
Ser – Serine  
Thr - Threonine  
STK – Ser/Thr Kinase  
STP – Ser/Thr Phosphatase  
ip – Immunoprecipitated  
Cov – Control of Virulence  
Wal – Wall Synthesis Regulating  
MalT – Maltose-Transport  
MalR – Maltose-Metabolism

**Key Words:** Reversible threonine phosphorylation, Response Regulator, SptR, Group A *Streptococcus*, Virulence

## ABSTRACT

GAS bacterium is a successful human pathogen that causes a variety of diseases of the skin and pharynx and results in about 500,000 deaths worldwide. The pathogenesis of GAS diseases is highly complex. GAS virulence is regulated by several two-component regulatory systems (TCS) constituted by a response regulator (RR) and its cognate histidine kinase (HK). HK autophosphorylates on its His residue and transfers the phosphate to the conserved Asp-residue of RR, which acts as a transcription factor and regulates the expression of specific genes. SptR/S (Spy874/875) is an important TCS that regulates the expression of many carbohydrate-metabolism and transport (CMT)-related genes. GAS mutants lacking SptR display down-regulated CMT-related genes and are avirulent. Previously, our lab discovered that similar phenotypic characteristics were also observed in GAS mutants when the gene encoding for ser/thr kinase (STK) was deleted. We therefore hypothesize that STK phosphorylates SptR and its phosphorylated form regulates the expression of CMT-related genes. STK phosphorylates threonine residues. Whether Thr-phosphorylation of SptR plays any role in the regulation of gene expression is not known. In *in vitro* phosphorylation assays, we found that the purified SptR serves as a substrate for STK and ser/thr phosphatase (STP)-mediated reversible phosphorylation. To confirm this finding *in vivo* and appreciate its physiological significance, we immunoprecipitated (ip) SptR from the whole cell lysate derived from the wild-type GAS, and previously characterized GAS mutants M1 $\Delta$ STK and M1 $\Delta$ STP using SptR specific antibodies. We confirmed isolation of the 27kDa ip-protein to be SptR by anti-SptR antibody and its threonine phosphorylation status by Anti-ThrP antibody by Western blot analysis. Our results showed decreased and increased anti-ThrP-specific binding with ipSptR from GAS mutants M1 $\Delta$ STK and M1 $\Delta$ STP, respectively, confirming that SptR indeed serves as a substrate for the STK/STP-mediated reversible Thr-phosphorylation. We thus demonstrate a novel virulence-related gene regulatory mechanism in GAS.

## INTRODUCTION

Group A Streptococcus (GAS, *S. pyogenes*) is an important gram-positive, chain-forming extracellular bacterium that colonizes the human pharynx and skin. It is responsible for causing a variety of diseases that range from uncomplicated pharyngitis and skin infections including impetigo, erysipelas, and cellulitis to scarlet fever and life-threatening invasive illnesses such as pneumonia, bacteremia, necrotizing fasciitis, streptococcal toxic shock syndrome, and nonsuppurative sequelae (acute rheumatic fever, rheumatoid arthritis, and glomerulonephritis) (Carapetis et al., 2005). Cases of severe GAS disease cause more than 500,000 deaths per year worldwide (Cohen-Poradosu and Kasper, 2007).

GAS possesses a variety of cell-associated and secreted virulence factors to remain as a successful pathogen. Surface-bound and secreted proteins expressed by GAS play important functions in bacterial adhesion, invasion, and proliferation. Various toxins including extracellular pyrogenic exotoxins A, B, and C, exotoxin F, and streptococcal superantigen SSA elicit the large non-specific activation of T cells and cause the production of inflammatory interleukins and cytokines (Cunningham, 2000). GAS also secretes proteins, toxins, and enzymes –including hydrolases and esterases– that play a role in tissue invasion and the degradation of proteins, nucleic acids, polysaccharides, and lipids. The expression of many of these proteins is dependent on bacterial growth and is controlled by two-component regulatory systems (TCS) composed of an environmental sensor, a sensor histidine kinase (HK), and a response regulator (RR) (Stock et al., 2000). GAS possesses 13 such TCS (Ribardo et al., 2004). TCS, thus, play a crucial role in regulating the genes responsible for successful colonization and infection by GAS.

Typically, HKs are signal-transduction transmembrane enzymes that monitor a cell's external environment and respond to extracellular cues, such as those produced by nutrients, chemoattractants, osmotic conditions, and quorums (Stock et al., 2000). Canonically, the HK autophosphorylates on a conserved histidine residue and transfers the inorganic phosphate group to the conserved aspartic-residue of the co-transcribing RR. Structurally, the RR

possesses two distinct domains, the N-terminal receiver and C-terminal DNA-binding domain. The activity of an RR is dependent on the aspartate phosphorylation on its N-terminal receiver domain, which brings substantial conformational changes to its carboxy-terminal DNA-binding domain (Stock et al., 2000). The Asp-phosphorylated RRs bind more efficiently to the promoter DNA and drive the transcription of the specific downstream genes. RR thus serves as a transcription factor.

Recently, in prokaryotes, an analogous alternative signaling mechanism mediated by serine/threonine kinases (STKs) and co-transcribing phosphatases (STPs) has been recognized and received importance as the mono-component regulatory system (Pereira et al 2011). STKs in bacterial pathogens including GAS have been linked to the regulation of key posttranslational-based cellular functions such as metabolic and developmental processes, stress response, biofilm formation, cell wall biosynthesis, sporulation, drug resistance, and virulence (Pereira et al., 2011). Previously, our laboratory has demonstrated that GAS ser/thr kinase (STK, SPy1625) and phosphatase-(STP, SPy1626)-mediated Thr phosphorylation and dephosphorylation respectively regulate the transcriptional function of the two other important RRs of TCS, CovR and WalR in GAS (Agarwal et al., 2012).

Among the 13 TCS found in GAS, three have been found to regulate GAS virulence: CovR/S, WalR/S, and SptR/S. CovR/S (control of virulence) regulates capsule and hemolysin production as a negative regulator (Churchward, 2007). WalR/S (wall synthesis regulating) regulates cell division and cell wall synthesis (Winkler and Hoch, 2008). Lastly, newly recognized SptR/S (for saliva persistence, SPy874/875) regulates many carbohydrate-metabolism and transport (CMT)-related genes (Shelburne et al., 2005). Although our laboratory has shown that STK/STP-mediated reversible phosphorylation regulates the functionality of CovR and WalR in GAS, it is unknown whether SptR is similarly regulated by STK/STP in GAS.

GAS lacks the Krebs cycle and hence, it uses glucose as the primary carbon source for its growth. In this thesis, I address the topic of GAS's successful pathogenicity, which causes a

variety of systemic invasive diseases in environments where glucose is unavailable. The importance of SptR lies in the crucial role that carbohydrate metabolism plays in GAS virulence regulation. SptR/S-regulated gene expression enables GAS to survive in glucose-depleted or non-glucose environmental niches and to utilize complex carbohydrates (non-glucose) present in human body fluids including saliva (hence saliva persistence). GAS mutants lacking SptR are avirulent and display down-regulated CMT-related genes (Shelburne et al., 2005). Similarly, GAS mutants lacking STK also display down-regulated CMT-related genes, but without any change in the expression level of SptR (Kant et al., submitted). This indicates that there is a direct physiological and biochemical link between STK function and CMT-related gene expression.

We therefore hypothesize that in the absence of STK, the functionality of SptR is altered. If our hypothesis is correct, STK should be able to phosphorylate SptR. STP should dephosphorylate SptR-P both *in vitro* and *in vivo*.

## **MATERIALS AND METHODS**

### **Bacterial Strains and Reagents:**

GAS mutants M1 $\Delta$ STK and M1 $\Delta$ STP lacking STK and STP, respectively, were made as described earlier (Jin and Pancholi, 2006; Agarwal et al., 2011) and obtained from our stock collection. Mutants were grown in Todd Hewitt broth supplemented with 0.5% yeast extract and spectinomycin (500  $\mu$ g/ml) or kanamycin (200  $\mu$ g/ml). These mutants were derived from the wild-type M1SF370 strain. *Escherichia coli* (DH5a or XL-1 and BL21 pLysS) strains were grown in Luria-Bertanii medium supplemented with or without ampicillin (100  $\mu$ g/ml). All other chemicals were obtained from Sigma Chemicals, unless otherwise indicated.

### **Cloning of the SPy874 gene and production of His-tag recombinant protein:**

The 669 nt open reading frame in the M1SF370 assigned as SPy874 was PCR amplified using forward and reverse primers containing Xho1 and BamH1 restriction sites, respectively.

Using the pGEM-T vector system and T4 ligase, the PCR product was ligated. The ligation mixture was then used to transform *E. coli* XL-1 strain by the chemical transformation method using competent cells and the colonies harboring pGEMT-SptR plasmid were selected on LB agar containing ampicillin. pGEM-SptR plasmid was then purified using Qiagen plasmid purification kit. As shown in figure 3, the plasmid was double digested with Xho1/BamH1 and the 669 bp fall-out fragment was then gel purified using Qiagen DNA gel purification kit. The purified DNA fragment was ligated between the Xho1/BamH1 site of double digested His-tag vector pET14b at 16°C overnight. The ligation mixture was then used to transform *E. coli* (XL-1 strain) *sptR* positive, (as determined by PCR using for forward and reverse primers as described above) and ampicillin resistant *E. coli* colony were selected. This strain was stored at -70°C until further use. Plasmid pET14b-sptR was then purified from *E. coli* XL-1 strain and subsequently used to transform BL21 DE3 (PlysS) strain.

#### **Purification of Recombinant SptR Protein:**

pET14b-SptR-transformed *E. coli* was grown overnight in LB broth containing ampicillin (100 µg/ml) under constant rotation at 37 °C. On the following day, 100 ml of LB broth was inoculated with 1 ml of overnight culture and grown to an optical density 0.3-0.4 (2- 3 hr) as described above and was induced with 1mM IPTG and further incubated for 3-4 h. The culture was then pelleted and suspended in binding buffer (50 mM Tris/HCl, pH 8.0, 0.15 M saline and 10 mM imidazole, protease inhibitor, DNase and RNase, 5 mM MgCl<sub>2</sub>). After repeated freezing and thawing, the cells were sonicated. The lysate thus obtained was centrifuged (10,000 g) for 20 min at 4°C. The supernatant and pellet were fractionated. Following this, the pellet was suspended in the above buffer containing 8M urea. The solubilized pellet was further centrifuged (10,000 g for 20 min at 4°C) and the debris-free supernatant was separated. Both native and denatured supernatant were subjected separately for purification as shown in the schematic diagram (figure 4).

Briefly, recombinant His-tag protein was purified by Ni-NTA-affinity column chromatography using three (50, 100, 250 mM) step-up gradients of imidazole in the Tris-buffer described above. Then, 2 ml of lysate was poured onto the column and fall through was recovered. The column was washed with 5 column volumes and subsequently eluted with 5 column volumes of each step up gradient buffer. Eluted fractions were examined for the presence of the 27 kDa SptR protein by SDS-PAGE electrophoresis.

#### **Raising Antisera against SptR:**

Rabbit polyclonal sera against the recombinant Histag-SptR was custom-raised (Lampire Biomedicals) employing the express protocol of 50 days of immunization as described on their website. Bleed out sera were checked for the high titer antibody against His-tagged SptR recombinant protein by western blot analysis, as described below.

#### ***In vitro* Reversible Phosphorylation of SptR by SP-STK and SP-STP:**

Phosphorylation assay was carried out using a reaction mixture containing SptR (2 µg), STK (0.2 µg),  $\gamma^{32}\text{P}$ -ATP (1 µCi) and 5 mM  $\text{MnCl}_2$  and incubated for 30 min at room temperature. A duplicate sample was then treated with SP-STP. The phosphorylation of SptR by SP-STK and SP-STP was analyzed by autoradiography.

#### ***In vivo* Phosphorylation:**

Determination of *in vivo* Thr phosphorylation status of SptR in the M1-Wt, M1 $\Delta$ STK, M1 $\Delta$ STP mutant strains was carried out as schematically presented in figure 5. Briefly, the whole lysate from the overnight cultures of the above strain was obtained by treating the washed bacterial pellet with recombinant phage lysis (amidase) to digest the cell wall in 50 mM Tris/HCl buffer, pH 8.0, DNase, RNase and protease inhibitor. Subsequently, a debris free supernatant was obtained after high-speed centrifugation (16,000 g for 20 min at 4 °C). This was then used as the starting material for immunoprecipitation. Anti-SptR was used to immunoprecipitate SptR present in the above-mentioned bacterial lysates. Immunoprecipitated SptR-Ab complex was fractionated by incubation with protein-A Sepharose beads that



specifically bound to the Fc-portion of anti-SptR antibodies. Using low centrifugation force, the proteinA-sepharose linked SptR-Anti-SptR ab complex was removed and thoroughly washed with washing buffer containing non-ionic detergent to remove non-specifically bound proteins (other than IgG molecules). The purified immunoprecipitated complex was then suspended in denaturing sample buffer for SDS-PAGE and western blot analysis.

#### **SDS-PAGE Western Blot Analysis:**

The proteins in the given samples were first suspended in 4x denaturing sample buffer (containing  $\beta$ -mercaptoethanol) and boiled for 2 min. The proteins in the samples were then resolved by 15% SDS-PAGE electrophoresis. For *in vivo* phosphorylation analysis, three samples (20  $\mu$ l each of wt, M1 $\Delta$ STK and M1 $\Delta$ STP mutant lysates) were run in triplicate to perform Coomassie staining and western blot analysis using anti-SptR and anti-threonine-P antibodies. The amount of immunoprecipitated SptR was determined by Coomassie stained gel. The integrity of the immunoprecipitated SptR was determined by the reactivity to anti-SptR antibody on Western immunoblot analysis. The status of Thr phosphorylation of immunoprecipitated SptR from the M1 wild-type and isogenic M1 $\Delta$ STK and M1 $\Delta$ STP GAS mutants was measured on Western blots using anti-Thr-P monoclonal antibody, which specifically reacts with Thr-P phosphorylated protein.

## **RESULTS**

Based on our stated hypothesis, we targeted SptR protein as the possible substrate for group A streptococcal serine/threonine kinase and phosphatase. Since we already have STK and STP-specific mutants derived from M1SF370, we investigated the presence of SptR homolog in M1SF370 genome for this investigation. We then cloned and expressed the corresponding gene and purified recombinant protein. Subsequently, we examined whether the recombinant SptR serves as a substrate for STK and STP under both *in vitro* and *in vivo* conditions.

**SPy0874 (SptR) is a response regulator of a novel Two-component regulatory system in the M1SF370 Genome:**

By BLAST and other bioinformatics tools and NCBI web resources, we found that the *sptR* gene is encoded by a 669 bp open reading frame *spy874*, located between nucleotides number 721,374 and 722,042. This gene is conserved in other completely sequenced M1 serotypes also (figure 6).

**Location of the *sptR* gene (*SPy\_0874*) in M1SF370 genome and its protein sequence:**

A 669 bp *sptR*-specific DNA fragment was PCR-amplified using forward and reverse primers containing Xho1 and BamH1 restriction sites respectively (see materials and methods). The *sptR*-specific PCR product was cloned in pGEM-T vector at the T-over hang site. After restriction digestion the PCR fragment was cloned in pET14b between Xho1 and BamH1 sites to allow in-frame expression of the *sptR* gene with upstream 6xHis-encoding tagged. The ligation product was then used to transform XL-1 *E. coli* strain and ampicillin-resistant colonies were selected to determine the presence of pET14b-SptR plasmid. The latter was purified from the positive clones and was used to transform BL-21 pLysS *E. coli* strain. Colony PCR was performed on several colonies. The *sptR*(+ve) *E. coli* clone was grown in LB-ampicillin medium until O.D. 0.35 and then induced with 1 mM IPTG for 4 h. Bacterial pellet was obtained from this induced clone. The pellet was lysed in 20 mM TRIS/HCl pH 8.0 containing 10 mM imidazole binding buffer.

The SptR was purified using Ni<sup>2+</sup>-NTA affinity column as described in the materials and method section. The proteins present in the fall-through, washed and eluted fractions were then resolved by SDS-PAGE and examined by Coomassie stain. As shown in figure 7, the SptR was eluted from the column when buffer containing 200 mM imidazole was employed. A substantial portion of this protein was also found in the pellet fraction. Hence, using a strategy of purifying denatured protein, a pellet suspended in 8M urea was used as the starting material. The purification pattern remained essentially the same as described above. However, for further

analysis, we dialyzed the denatured protein, employing multiple dialysis using a buffer system with sequentially reducing urea content i.e. TRIS buffer with 4 M followed by 2 M, 1 M and 0 M urea. This allowed us to refold the protein. The purified protein was then used for *in vitro* phosphorylation assays.

### ***In vitro* Phosphorylation of SptR**

SP-STK is a transmembrane protein (Jin and Pancholi, 2006). Earlier attempts from our lab have shown that the yield of the whole protein is low and degrades very rapidly. However, the kinase domain (hereafter referred as STKK) is a soluble enzymatically active fragment and can easily be purified (Jin and Pancholi, 2006)

*In vitro* phosphorylation assay was carried out using a reaction mixture containing SptR (2 µg), STKK (0.2 µg),  $\gamma$ -<sup>32</sup>P-ATP (1 µCi) and 5 mM MnCl<sub>2</sub> and incubated for 30 min at room temperature as described in material and methods. As shown in figure 8, STKK (36 kDa fragment) autophosphorylated in the presence of  $\gamma$ -<sup>32</sup>P-ATP and also phosphorylated SptR (27 kDa). However, when this reaction mixture was treated with recombinant STP (27 kDa Protein), there was substantial decrease in phosphorylation due the dephosphorylation reaction mediated by STP. We also observed that with extended incubation for 1hr, or by increasing the amount of STP with the same 30 min of incubation, both STKK and SptR were completely dephosphorylated (data not shown). Notably, SptR in the absence of STK but in the presence of only ATP was not phosphorylated, indicating that the reversible phosphorylation of SptR was mediated by STKK and STP.

### ***In vivo* Phosphorylation**

To determine whether the observed *in vitro* STK-mediated phosphorylation of SptR indeed occurs *in vivo* and thus physiologically relevant, we isolated SptR from the wild-type with intact STP and STK expression and mutant GAS strains that lack either STK (M1 $\Delta$ STK) or STP (M1 $\Delta$ STP) and determined their phosphorylation status. SptR was isolated from the lysates of

these strains by employing the immunoprecipitation method as described in materials and methods, using polyclonal anti-SptR antibody.

As shown in figure 9, the Coomassie stain confirmed the presence of the immunoprecipitated SptR from the wild-type, and isogenic M1 $\Delta$ STK and M1 $\Delta$ STP mutants and Western blot analysis with Anti-SptR antibody confirmed the specificity and integrity. The comparison of the densitometric analysis of Coomassie-stained and corresponding anti-SptR reacting protein bands revealed equal amount of SptR proteins in all the GAS strains. The reactivity of anti-ThrP antibody revealed a significant decrease in the reactivity of SptR recovered from the M1 $\Delta$ STK vs. M1 $\Delta$ STP, indicating that in the absence of STK, threonine phosphorylation of SptR was adversely affected. This correlates with our earlier finding showing poor growth of M1 $\Delta$ STK in complex carbohydrates (Kant S. et al 2013 unpublished data).

## DISCUSSION

Knowing that GAS uses glucose as a primary source of energy and growth, in the present investigation, we have addressed the important topic of GAS's survival and proliferation to remain as a successful pathogen in a host environment where glucose concentration is either highly depleted or absent. SptR/S is an important TCS that regulates the expression of many carbohydrate-metabolism and transport (CMT)-related genes including those regulating maltose-transport (MalT), maltose-metabolism (MalR), and GAS virulence. SptR is up-regulated in GAS when grown in saliva, of which maltose is a major constituent (Shelburne et al., 2005). GAS mutants lacking SptR are non-virulent and unable to grow in saliva. SptR function is likely regulated by its cognate SptK (SPy 875). Previously, our lab observed similar phenotypic characteristics revealing down-regulation of MalR and MalT in GAS mutants when the ser/thr kinase (STK) encoding gene was deleted (Kant S. et al 2013 unpublished data). Thus far, it is not known that SptR can be phosphorylated by other non-canonical kinases. In the present investigation, we establish that SptR, in addition to the canonical pathway as reported before

(Shelburne et al., 2005), is also phosphorylated by SP-STK. SP-STP (co-transcribing ser/thr phosphatase), on the other hand, dephosphorylates Thr-phosphorylation ( $-P^{\text{Thr}}$ ) both *in vitro* and *in vivo*. It is so far not known where SptR binds and how it regulates carbohydrate metabolism.

For future studies, we propose that SptR- $P^{\text{Thr}}$  may bind to the upstream promoters of *malR* (*PmalR*) and *malT* (*PmalT*) more efficiently and subsequently regulate the transcription of *malR* and *malT*. Our ongoing studies will address this hypothesis and may reveal the mechanism of STK/STP mediated fine-tuning of response regulator function by post-translational modification of Thr residues. The identification of specific Thr residues in the SptR by mass spectrometry may broaden our understanding about the STK/STP mediated regulation of carbohydrate metabolism and how GAS remains a metabolically fit and successful pathogen.

## **PRESENTATIONS**

The Natural and Mathematical Sciences Undergraduate Research Forum

The Ohio State University, 2012

Presented by: Alisha Kamboj, Rebekah Lantz

Awarded "Outstanding Project"

The OSU Denman Undergraduate Research Forum

The Ohio State University, 2012

Presented by: Rebekah Lantz

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## FIGURE LEGENDS:

**Figure-1.** Group A Streptococcus (GAS, *S. pyogenes*) morphology and health consequences of GAS colonization in the human body. (A) The gram-positive, chain-forming characteristics of Group A Streptococcus (GAS, *S. pyogenes*) are visible under a microscope after gram staining (Chamberlain, 2013). (B) Throat infections, forming a white patch in the pharyngeal area, as well as tonsillitis, indicated by enlarged tonsils, are the result of GAS colonization of the human pharynx (Kaneshiro et al., 2012). (C) Necrotizing fasciitis (infected leg) is also caused by GAS and is known as flesh-eating disease. It is a rare infection that penetrates into the deeper layers of the skin (Hu, 2002).

**Figure-2.** Classical prokaryotic two-component systems regulation. The schematic diagram above displays the basis of our hypothesis as to how our recently identified SptR (SPy0874) and SptS (SPy0875) is likely to function based on the classical prokaryotic TCS functioning. Upon autophosphorylation of HK on a conserved histidine residue, the phosphate group is transferred to the conserved Asp-residue found on the RR. Dimerization followed by the initiation of transcription occurs once the DNA binding domain of the SptR binds to the DNA binding site.

**Figure-3.** Preparation of pET14B-SptR plasmid and transformation in BL21 *E. coli* strain. Schematic diagram showing a stepwise strategy for the expression of sptR gene by was cloning the PCR product in pET14B vector and expression in BL21 *E. coli* strain under IPTG induction.

**Figure-4.** Purification of recombinant SptR protein. Schematic diagram depicting stepwise strategies (native and denaturing) for the purification of IPTG-induced recombinant protein by affinity chromatography employing Ni-NTA solid-phase column.



**Figure-5.** *In vivo* Thr phosphorylation status of SptR in the M1-Wt wild-type and M1 $\Delta$ STK, M1 $\Delta$ STP mutant GAS strains. Schematic diagram depicting a stepwise strategy to isolate differentially phosphorylated *in vivo* expressed SptR in different GAS strains using anti-SptR antibody and Protein A-Sepharose-4B.

**Figure-6.** Location of the *sptR* gene (*SPy\_0874*) in M1SF370 genome and its encoded protein sequence.

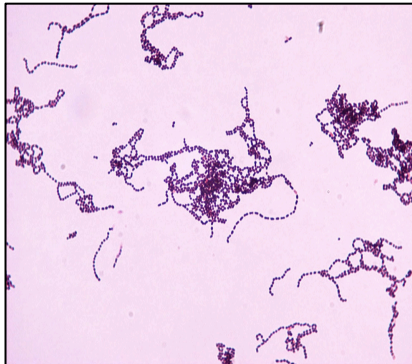
**Figure-7.** Purification of recombinant His-tag SptR. SDS-PAGE shows the presence of 27kDa protein in the eluted fractions on Ni-NTA column. Identification of SptR by anti-SptR antibody. FT- Fall-through sample. Wash- fraction collected with buffer containing 50mM imidazole. Elution- fractions collected with buffer containing 200mM imidazole. The last lane shows the reactivity of SptR with anti-SptR antibody.

**Figure-8.** *In vitro* phosphorylation of SptR. Autophosphorylation of SptR was carried out by SP-STK using  $^{32}\gamma\text{P}$  –ATP. SptR-<sup>ThrP</sup> was dephosphorylated by SP-STP.

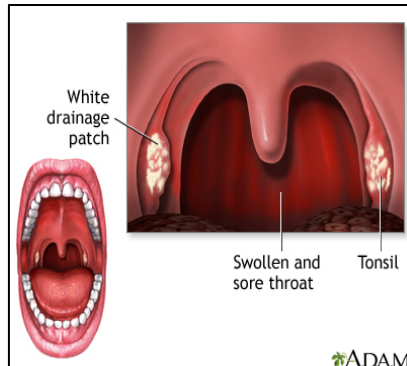
**Figure-9.** *In vivo* differential phosphorylation status of SptR in the wild-type M1SAF370 and  $\Delta$ STK and  $\Delta$ STP GAS mutants. Immunoprecipitated SptR from different GAS strains as revealed by Coomassie stain (A) and its reactivity with anti-SptR antibody (B) and anti-Thr-P monoclonal antibody (C) as revealed by chemiluminescence-based visualization assay. Quantitative analysis of the antibody-reactive bands as determined from three independent experiments and analyzed statistically by Student's T-test. P value less than 0.05 was treated as significant difference.

Figure 1. Group A Streptococcus (GAS, *S. pyogenes*) morphology and health consequences of GAS colonization in the human body.

**A**



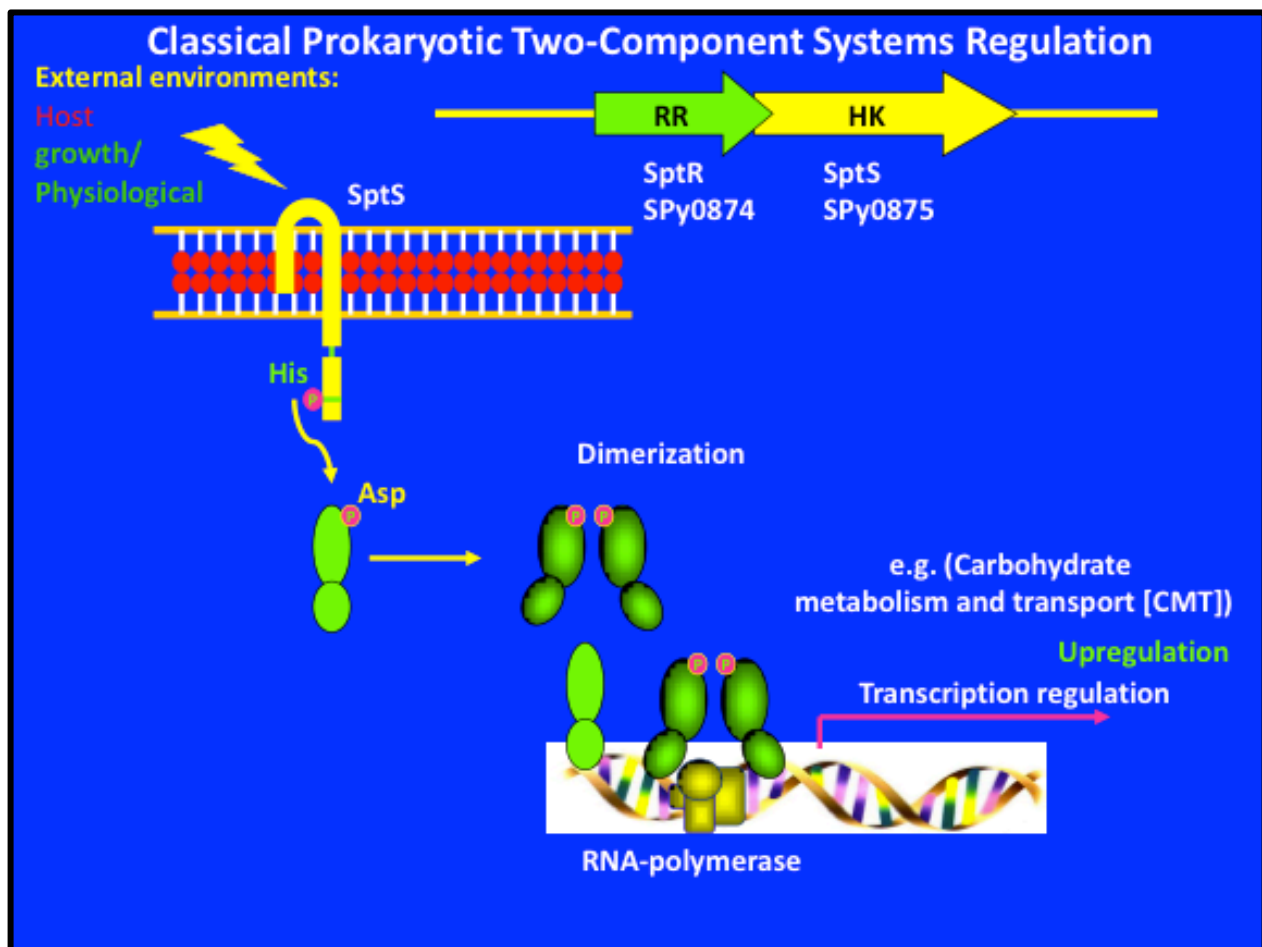
**B**



**C**



Figure 2. Classical prokaryotic two-component systems regulation



The diagram illustrates the construction and expression of the pET14B-SptR plasmid. It begins with the pGEM<sup>®</sup>-T Vector (3000bp) and a DNA fragment containing the *sptR* gene. The *sptR* gene is inserted into the pGEM<sup>®</sup>-T Vector using XhoI and BamHI restriction enzymes. The resulting pET14B-SptR plasmid (~5.34 kb) is then transformed into XL-1 *E. coli* strain. The plasmid is prepared and then transformed into BL21 *E. coli* strain for SptR expression.

Figure 4. Purification of recombinant SptR protein

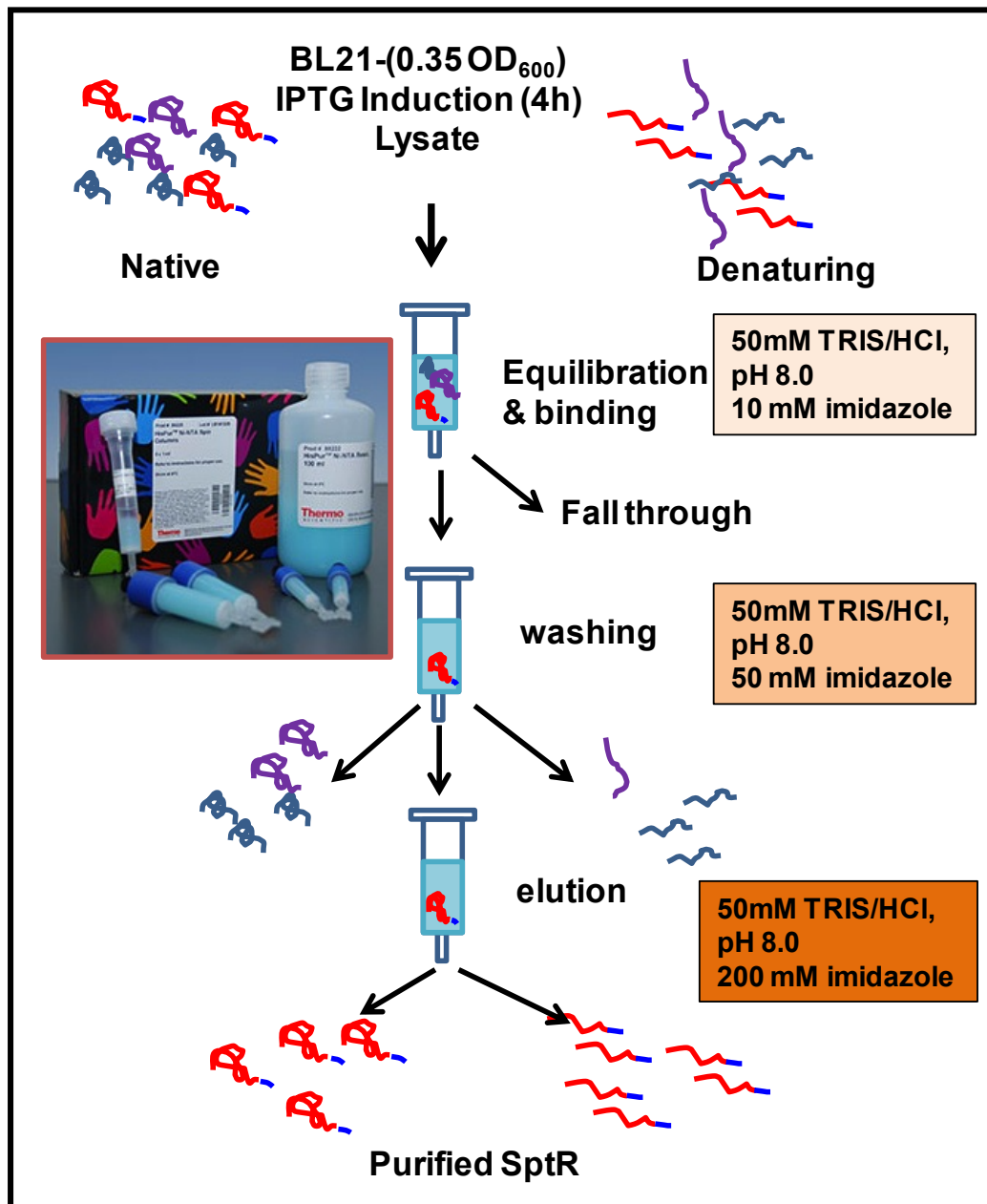


Figure 5. *In vivo* Thr phosphorylation status of SptR in the M1-Wt, M1 $\Delta$ STK, M1 $\Delta$ STP mutant strains

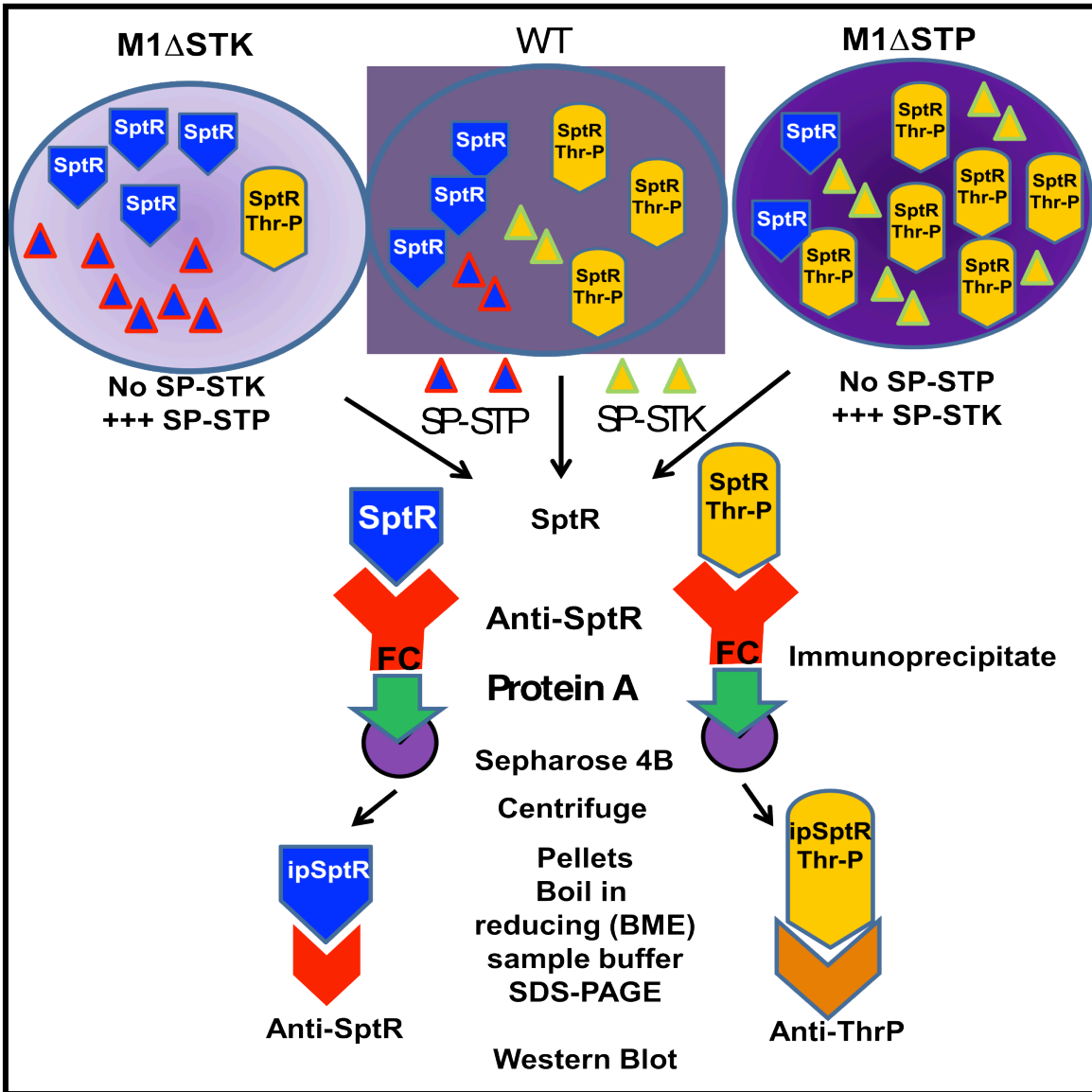


Figure 6. Location of the *sptR* gene (SPy\_0874) in M1SF370 genome and its encoded protein sequence.

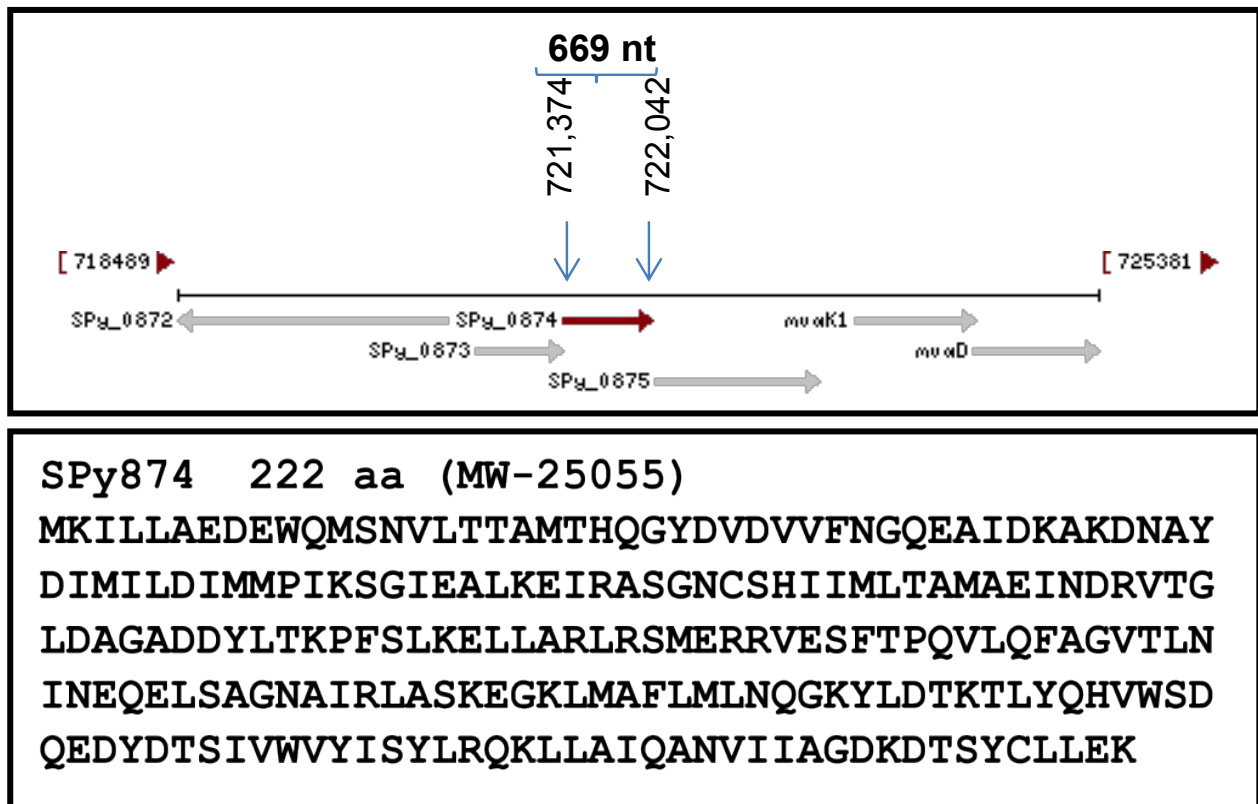


Figure 7. Purification of recombinant His-tag SptR.

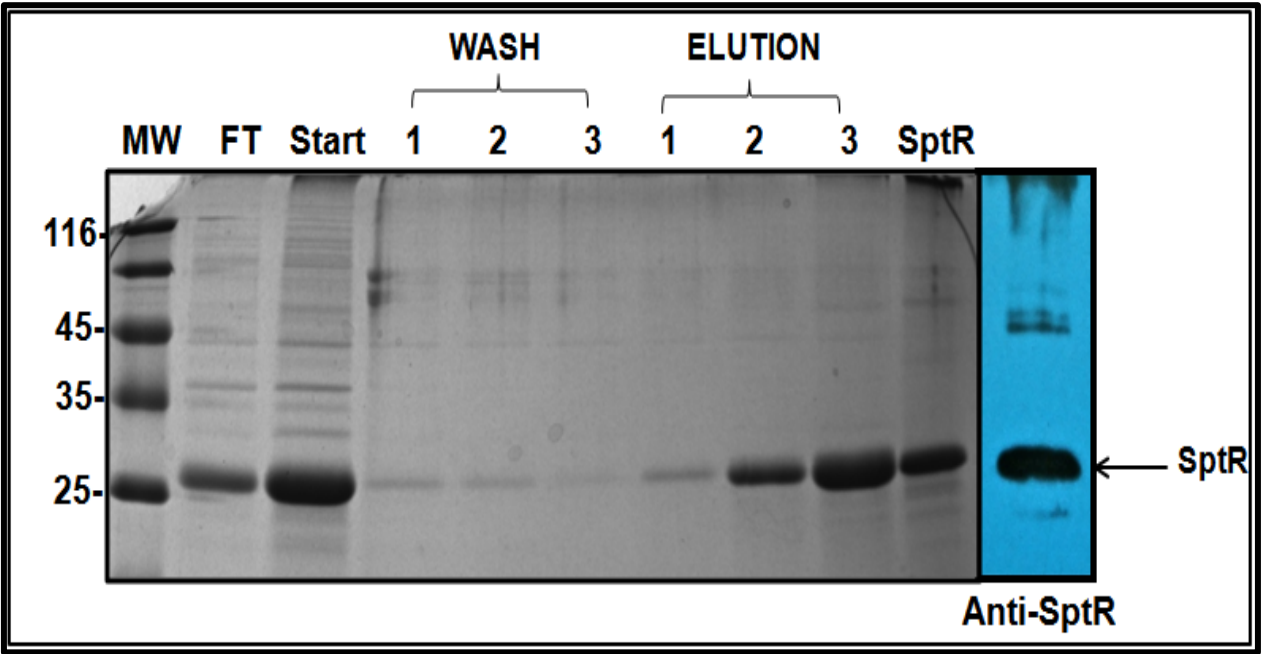




Figure 8. *In vitro* phosphorylation of SptR

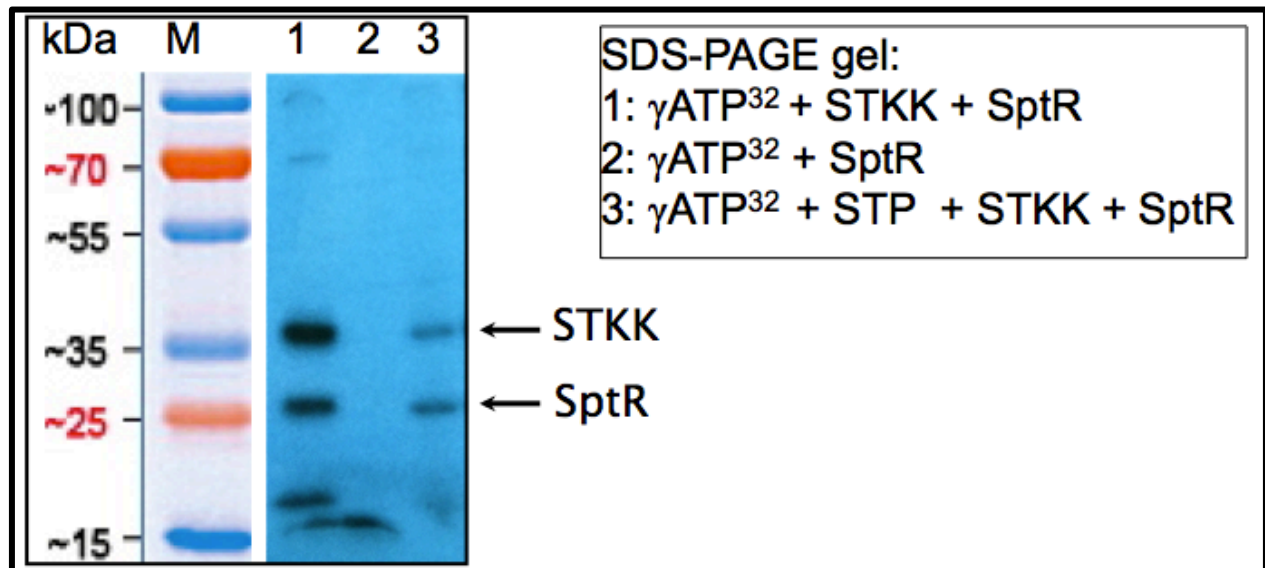


Figure 9. *In vivo* differential phosphorylation status of SptR in different GAS strains.

